Optical manipulation of liposomes as microreactors

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Abstract

We present an all optical method to manipulate and fuse giant liposomes, typically 10 μm in diameter. Optical tweezers are used to trap two individual liposomes, which are then brought into contact. A single pulse of ultraviolet laser light focused on the adjoining membranes induces the fusion between the liposomes. As a consequence, the chemical reagents encapsulated in the two liposomes mix and a chemical reaction takes place.

INTRODUCTION

The cell is arguably the basic building block of living organisms and the fundamental chemical processing plant for sustaining life. Inside the cell, complex chemical reactions typically take place in small volumes and often involve only a small number of molecules. Such a "nano"-environment raises questions about the applicability of bulk chemical assays towards understanding and, ultimately, influencing or controlling cellular processes. For example, the constant energy flow essential for most biological processes suggests that non-equilibrium chemistry plays an important role in maintaining life. Bulk assays, which often provide only equilibrium results, may be inadequate in helping to identify the relevant processes in biological systems.

The need for studying cellular processes, evaluating cellular response as well as performing high-throughput reactions in the pharmaceutical industry has prompted the rapid development of ultra-small volume analytical systems such as microfluidic devices, microarrays and nanovial arrays. Nanovials offer the advantage of parallel sample analysis in a similar manner to the more common microtiter format. Various techniques [1, 2, 3, 4, 5, 6, 7] exist to create open volume nanovials, which hold nanoliter to femtoliter of reagents. In these systems, however, control of evaporation is a major issue and individual reaction conditions such as temperature or incubation time are difficult to control independently [1]. While such approaches offer many insights into the chemistry of reactions at small volumes, they do not constitute biologically relevant nano-environments.

We report here on the use of giant (cell-sized) liposomes [8] as self-enclosed nanovials to perform ultra-small volume chemical reactions [9, 10, 11]. As containers, liposomes are similar to cells in that they are composed of phospholipids that self-assemble in water forming an internal aqueous cavity that is isolated from an external aqueous solution. In our samples liposomes range in size from 0.5 μm to 50 μm in diameter, enclosing a volume that is measured in femtoliters to picoliters . The liposomes stably entrap molecules while bathed in an aqueous solution allowing control over the internal environment and eliminating concerns about evaporation. Liposomes can function as moveable containers, and reactions may be performed inside them in a controlled manner. Reactions inside a liposome may be initiated by encapsulating all reactants in a single liposome upon formation, by selectively diffusing some reactants through the membrane from the external bathing solution, or by fusing two liposomes together containing different reactants.

In this paper, we demonstrate the use of giant liposomes as individually-controlled sub-picoliter reaction containers with which to evaluate chemical and biomolecular kinetics. We have focused on encapsulation of analytes inside stable liposomes, controlled manipulation and movement of liposomes, and fusion of liposomes for mixing of the encapsulated reactants and their chemical reaction. Unique to this work is the use of only optical tweezers to bring two liposomes into intimate contact and the use of a high energy ultraviolet (UV) laser pulse to initiate their fusion, which consequently allows their contents to mix and react. Fusion using electroporation of two liposomes for a controlled chemical reaction was reported [9, 10, 11, 12]. In this method a high voltage electrical pulse applied to an electrode placed in proximity to the lipid membrane destabilizes the entire liposome membrane. In contrast, in our work the UV pulse, which initiates the fusion, is highly focussed and disrupts the lipid membrane only very locally. As a result, the possibility of leaking small encapsulated molecules during the fusion decreases considerably. In addition, our all-optical approach has the advantage that the liposomes can be fused anywhere in the sample chamber, in contrast to electroporation, where fusion is constrained to take place at the location fixed by the position of the electrode.

MATERIALS AND METHODS

Liposome preparation The liposomes were prepared by a reverse phase evaporation method [13]. The lipid 1-Palmitoyl-2-oleoyl-Sn-glycero-3-phosphocholine (POPC), purchased from Avanti Polar Lipids, Inc. of Birmingham, AL, USA [14], was dissolved in chloroform at a concentration of 30 mM. From this solution 50 μL were added to a 50 mL round bottom flask. Then 3 mL of aqueous phase (10 mM Hepes buffer with 11 mM $MgCl_2$, pH 7.4) was carefully added along the side of the flask wall using a pipette. The organic solvent, chloroform, was then removed in a rotary evaporator (Büchi, Switzerland, Model R-200) under reduced pressure. The final pressure was 40 mm of Hg at 40 $^\circ$C while rotating at 40 rpm. Under these conditions, boiling of chloroform was observed. After two minutes of evaporation an opalescent fluid was left in the flask, which contained the liposomes suspended in the Hepes buffer solution.

The chemicals to be encapsulated in the liposomes were added to the buffer solution before the evaporation of the organic solvent was carried out. For the experiment in which we demonstrate mixing of the reactants after fusion, we used 1 mM sulforhodamine B (Molecular Probes, Inc. of Eugene, OR, USA) in Hepes buffer for encapsulation. Subsequently, the liposomes prepared in the presence of dye were "washed" to remove the dye from the solution in which the liposomes were suspended. The washing procedure consisted of multiple cycles of centrifugation and subsequent resuspension of the pellet in dye-free buffer solution. For observation under the microscope and fusion experiments, samples were prepared in a reservoir on a microscope slide with approximately 100 μL of Hepes buffer, and 10 μL of each type of liposomes in solution, *i.e.* liposomes containing only buffer and liposomes containing sulforhodamine B.

For the experiment in which a chemical reaction occurs inside the final liposome, we fused two types of liposomes: one that contained 10 μM calcium chloride (Sigma, St. Louis, MO) and one that contained 1 μM fluo-3-pentaammonium salt (Molecular Probes), both in Hepes buffer solution. Experiments were carried out with samples prepared with 100 μL of Hepes buffer, 10 μL of 100 mM ethylenediaminetetraacetic acid (EDTA)(Sigma) and 5 μL of each type of liposomes.

Optical setup In order to observe, trap and fuse the liposomes we used an inverted microscope (Zeiss, Germany, Axiovert S100) equipped with the necessary dichroic mirrors and optical filters (all from Omega Optical, Brattleboro, VT) to accomodate all different wavelengths of light involved. Optical trapping of single liposomes has been demonstrated previously [9, 10, 11]. In our experiments, however, trapping of the liposomes was done using dual optical tweezers [15], with light from a Nd:YAG laser (Quality Electro-optics, Ltd, Tucson, AZ) at a wavelength of 1064 nm. Both optical traps were mobile, *i. e.* they could be used to move the trapped liposome in the focal plane of the microscope objective

lens, and thus appropriately position the liposomes before the fusion was initiated. Generally, we used approximately 140 mW of laser power per trap, as measured before the 100X, N.A.=1.3 oil immersion objective lens (Zeiss, Plan-Neofluar), which was used to focus the light. The fusion was induced by an "optical scalpel" [16], which consists of a single pulse of ultraviolet (UV) light from a UV laser (Continuum, Santa Clara, CA) functioning in triggered single pulse mode. The wavelength of the UV light was 355 nm, the pulse length 5 ns and the energy per pulse used was approximately 2 mJ.

All laser light, for trapping, fusion and fluorescent excitation passed through the same objective lens. The IR and UV light were focussed by the microscope objective lens inside the sample, while the fluorescence excitation light uniformly illuminated the sample. Alignment of the UV beam was performed at very low intensity by exciting fluorescence in a sample of coumarin dye (1 mM). The convergence of the beam was adjusted such that the focus of the UV beam lay further inside the sample (by approximately 5 μm) than the foci of the IR beams. This was done in order to insure that the UV beam was focussed in approximately the same plane as the contact point of the membranes of the two liposomes that were to be fused. (In the inverted microscope the liposomes are trapped in the optical tweezers slightly above the focus of the trapping beams.) Fine tuning of the focal plane of the UV-beam was made on a sample contaning liposomes. For all fusion experiments, the position of the UV laser was kept fix and the liposomes were manipulated such that their membranes touched at this position.

The microscope was equipped with two cameras, one CCD camera (Cohu, San Diego, CA) employed for white light video microscopy and one intensified CCD camera (Roper Scientific, Trenton, NJ, Model I-PentaMAX) used in gated mode for fluorescence detection. A tunable argon ion laser (Melles Griot, Carlsbad, CA) was used for fluorescence excitation. In order to minimize photobleaching, exposure to the fluorescence excitation light was synchronized to the exposure time of the intensified CCD camera. We used an exposure time of 100 ms or less, depending on the brightness of the emitted fluorescence, which in turn depended strongly upon the characteristics of the dye molecules and their concentration inside the liposome.

RESULTS AND DISCUSSION

Characterization of the liposome samples The liposome samples, obtained using the reverse phase evaporation method described above, contained mostly multilamellar liposomes, whose sizes range from approximately 0.5 μm to 50 μm in diameter (see Fig. 1a). Although, under video microscopy many liposomes appeared as having a single outer layer (see Fig. 1b), their membrane stability towards thermal fluctuations [17] and fusion properties, which we will discuss later, suggested that the membrane was likely composed of multiple lipid bilayers. The

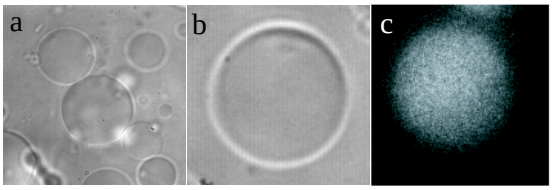

Figure 1: Bright field microscopy images of liposomes prepared by the reverse phase evaporation method. a) A typical sample (image size 48 μm x 48 μm). b) A multilamellar liposome (11 μm diameter) that is difficult to distinguish from a unilamellar liposome under video microscopy c) Fluorescence image of a liposome (approximately 4 μm diameter) containing sulforhodamine B dye.

liposomes were generally quite stable and could be kept for weeks at room temperature. They also survived several washing and centrifugation cycles. This feature was important when incorporating different chemicals, and in particular various fluorescent dyes inside the liposomes, since the water soluble chemicals were added to the aqueous solution before liposome formation by reverse phase evaporation (see Materials and Methods above). Figure 1c shows an image of a liposome containing sulforhodamine B dye

that has undergone the described washing procedure. The fluorescence of the background due to dye suspended in the bathing solution could be practically eliminated.

Liposomes containing fluo-3 pentaamonium salt (fluo-3 dye) and liposomes containing calcium chloride were not washed, because both molecules were found to readily cross the liposome membrane. Any difference in osmolarity between the inside and the outside of the liposome would favour leakage of calcium ions across the membrane in order to equilibrate the ion concentration. For this reason, we prepared on the microscope slide a sample with the two types of liposomes, Hepes buffer and EDTA. The EDTA, which is not membrane permeable, will bind with high affinity any ions, calcium or magnesium, on the outside of the liposomes. The effect is two-fold: calcium is hindered from crossing the membrane into the fluo-3-containing liposomes, and the number of free calcium or magnesium ions available for chelating by any fluo-3 molecules in the bathing solution is reduced. In the first case the fluorescence of the fluo-3-containing liposomes before fusion is kept low, while in the second case the background fluorescence is reduced significantly due to the relatively high concentration of EDTA in the surrounding medium. EDTA was chosen because it does not fluoresce, neither by itself nor when binding the ions.

We note here that unilamellar liposomes are very difficult to trap using conventional optical tweezers [18]. The membrane of such liposomes is only approximately 3 nm thick and it is often subject to thermal fluctuations. Therefore, from the optical manipulation point of view, it is preferable to use multilamellar liposomes, which have a more stable membrane and can be trapped in a reliable fashion.

Fusion of liposomes Figure 2 illustrates schematically the fusion of two liposomes containing two different reagents A and B. Each liposome is trapped in a separate optical tweezers and positioned such that the membranes of the two liposomes are in contact. At the contact point, a focused UV laser disrupts the membranes and the lipid bilayers reorganize spontaneously forming a new, larger liposome in which reagents A and B can mix.

In practice, we first identified a liposome in the sample and trapped it with one of the optical tweezers (we did not observe any deformation of the liposome due to the trapping). We then searched the sample for a second liposome of comparable size and trapped this liposome in a second optical trap. We positioned the two trapped liposomes until we observed a slight deformation of their membranes. A single UV light pulse was then applied at the contact point and we observed the subsequent formation of a single larger liposome. Figure 3 shows a sequence of four images taken by video microscopy that illustrate the fusion process between two liposomes, which do not contain different encapsulated reagents. Typically the size of the liposomes that we fused was on the order of 5-15 μm in diameter.

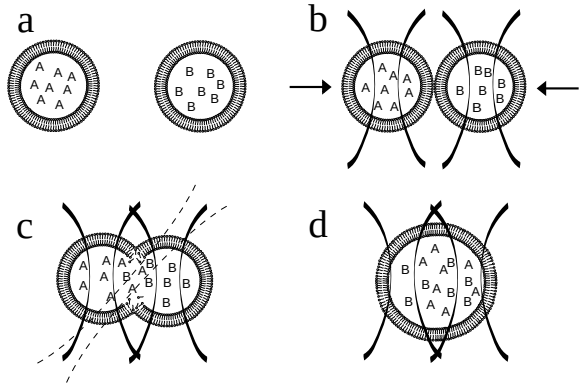

Figure 2: Schematic representation of the procedure for the trapping and fusion of two liposomes, containing different chemicals. a) Two liposomes, one containing reagent A and the other one containing reagent B, are identified in the sample. b) The two liposomes are trapped in separate optical tweezers and translated such that their membranes come into contact. c) Fusion is initiated by a pulsed UV laser, which disrupts the membranes of both liposomes at the contact point. d) The membranes repair spontaneously by forming one larger liposome in which the reagents A and B mix.

For all fusions that we recorded, we computed the enclosed volume and the surface area of the liposomes before and after fusion, by measuring their diameters

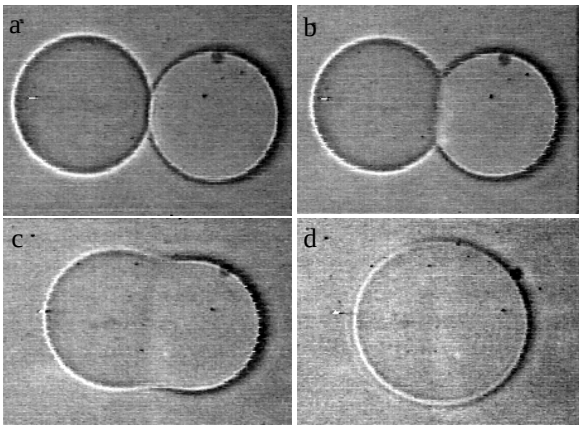

Figure 3: Fusion of two liposomes. The images are recorded by video microscopy. The fusion was initiated by the UV laser at the time when the first image was recorded. The next two images capture the progression of the fusion process at 132 ms and 264 ms, respectively. The last image, recorded at 528 ms, shows one single large liposome formed as a result of the fusion.

and assuming a spherical shape. In order to avoid errors due to the slight deformation of the liposome images at the contact point, we generally measured the diameters along the direction perpendicular to the direction determined by the contact point and the centers of the two liposomes to be fused. (In Fig. 3 for example, the diameters were measured along the vertical direction.) In order to verify whether the volume or the surface were conserved during fusion, we compared the measured volumes and surface areas of the fused liposome to the values of volume and surface expected in case of volume and surface conservation, respectively. The expected volumes (surfaces) were calculated as the sum of the volumes (surfaces) of the two liposomes prior to fusion. In about 10% of the fusions it remained unclear whether surface or volume was conserved. In the remaining 90% of the fusions, the volume appears conserved and the histogram in Fig. 4 shows the distribution of the difference between the measured and expected volumes of the final liposome, normalized to the measured volume. The spread of the distribution in Fig. 4 is mainly due to errors in the estimation of the diameter of the liposomes. We note that if the surface were perfectly conserved, the difference in volume as presented in Fig. 4 would be approximately 39%, which would be well outside the range of the data represented in Fig. 4.

The observation that the volume is conserved during the fusion suggests that the membranes of the liposomes were multilamellar, rather than unilamel-

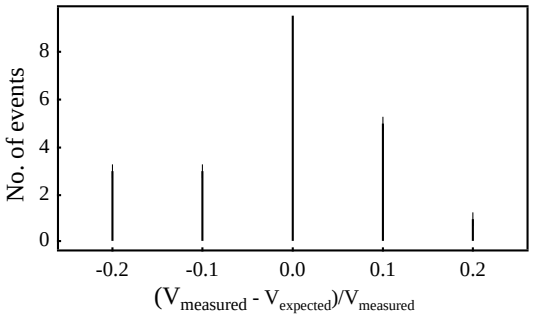

Figure 4: Histogram of the difference between the volume of the final liposome and the expected volume relative to the measured final volume, assuming that the volume is conserved.

lar. Unilamellar membranes are likely to remain unilamellar during the fusion process [19] and therefore one would expect the surface area to be conserved. Hence, without the uptake of additional fluid from the surrounding medium, the unilamellar liposome formed by fusion would not be spherical, but can assume a variety of shapes to conserve both area and volume. Since disruption of the lipid membranes by our pulsed UV laser is localized and the fusion occurs at the contact point between the two liposomes, the final liposome would most likely be prolate-dumbbell to sausage shape. Such shapes are local minimum energy configurations within the bilayer coupling model [20] and therefore are stable. In our experiments, we found that the final liposome was spherical and that the volume remained constant during fusion. Therefore, we speculate that the membranes of the liposomes that were fused, as well as the ones of the re-

sulting liposomes, were composed of several bilayers, which reorganized after disruption in such a way as to minimize the intake of fluid from the surrounding medium. It is also possible that as a consequence of the fusion a membrane reservoir [21] were formed, which contains the "excess" membrane. One noteworthy advantage of the volume conservation during fusion is the fact that the reactants are not diluted by the uptake of surrounding fluid and hence micro-reactions can occur with controlled quantities of reagents.

We observed a range of times for the fusion process. The shortest fusion we observed happened in 66 ms, while the longest fusion time measured was 1.5 s. The average fusion was found to last approximately 500 ms. We suspect that the fusion time may be related to the number of lamellae that form the liposome.

In order for the liposomes to be useful as microcontainers for controlled reactions, their contents must mix after fusion. We demonstrated that two reagents encapsulated in two liposomes mix as a consequence of the fusion. Figure 5 shows a series of four

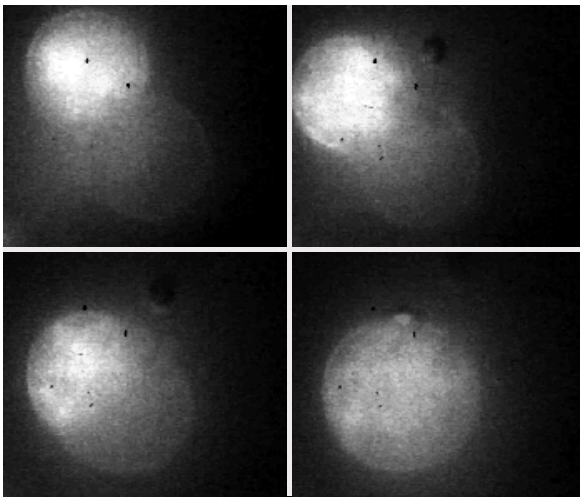

Figure 5: Fusion of two liposomes, one containing sulforhodamine B and the other one containing buffer only.

images recorded during the fusion of two liposomes, one which was filled with sulforhodamine B dye and the other one which contained only buffer, without dye. One can see that the fluorescence intensity after the fusion has decreased due to dilution of the dye with buffer from the second liposome. We verified that in this fusion, too, the volume of the two liposomes was conserved. The fluorescence distribution in the final image is fairly uniform indicating that the dye molecules have distributed themselves throughout the enclosed volume. Using a value of $2\times10^{-10}\,\mathrm{m}^2/\mathrm{s}$ for the diffusion coefficient of sulforhodamine B [22], we estimate that the time for the dye molecules to diffuse a distance equal to the radius ($5\,\mu$m) of the final liposome is approximately 60 ms. This time is comparable to the time between video frames and is consistent with the observed mixing time.

In another series of experiments we showed that liposomes can indeed be used as microreactors. The two reactants, fluo-3 dye and calcium ions were encapsulated in two liposomes that were fused. The upper two images in Fig. 6 show the bright field image and the corresponding fluorescence image of the two liposomes before fusion. The liposome containing calcium is almost dark, while the liposome containing fluo-3 fluoresces somewhat. Fluo-3 chelates any ions available in solution and fluoresces. However, the increase in fluorescence is considerably larger with calcium ions because they are bound with highest affinity. In our experiments the Hepes buffer contains magnesium, which is critical to the formation of the giant liposomes, and is responsible for the fluorescence of fluo-3-containing liposomes before fusion. In much of our data liposomes containing calcium ions appear to be completely dark and practically indistinguishable against the background. In other data, for example in the image shown in Fig. 6, these liposomes fluoresce weakly. We attribute this fluorescence to dye molecules leaking into the calcium containing liposomes since the lipid bilayer membrane is known to be permeable to fluo-3 dye. The bottom two images of Fig. 6 show the final liposome formed upon fusion. The total observed fluorescence increased by approximately 60% after fusion as compared to the value before fusion.

We note that the field of view as well as the magni-

fication of the images taken in bright field video microscopy and in fluorescence microscopy are slightly different. In addition, in the fluorescence images the background was subtracted using a background image that was recorded in a region of the sample that did not contain liposomes. We obtained the total fluorescence by integration of the image after background subtraction.

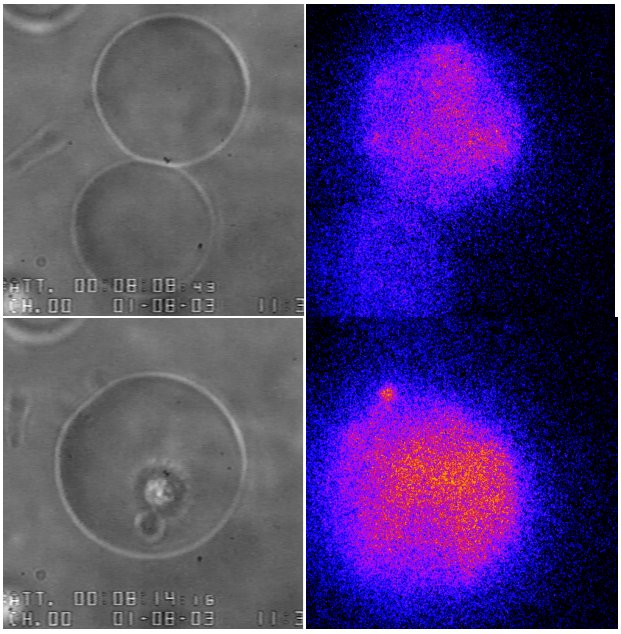

Figure 6: Fusion of two liposomes, one containing fluo-3 dye and the other one containing calcium ions. The bright field video microscopy images and the fluorescence images recorded simultaneously before and after the fusion was initiated (upper and lower images, respectively). After the fusion the fluorescence increases as a consequence of the reaction in which fluo-3 chelates the calcium ions.

In conclusion, we demonstrated an all-optical method that allows manipulation and fusion of giant liposomes. The setup we constructed is highly flexible and allows us to simultaneously trap two liposomes, without significantly deforming them and without exerting excessive stress on their membranes, as is the case when employing other techniques that allow manipulation and fusion of individual liposomes, such as microelectroporation [23] or microinjection [24]. With our procedure the liposomes can be moved within the medium and positioned relative to each other such that their membranes touch. Fusion of the liposomes is initiated by a very short pulse of UV laser light that is highly focused thus ensuring a minimal disruption of the membranes of the original liposomes. Hence this technique may be a versatile way to study liposome fusion in general.

We proved that liposomes can be employed as microreactors, *i.e.* a chemical reaction between reactants in different liposomes occurs upon their fusion. Implementing our setup in a microfluidic [25] flowcell environment, would further increase the flexibility of the system. In addition, it would minimize the effect of leakage across the liposome membrane from and into the bathing medium and thus reduce background fluorescence even more. In our experiments we found that volume is conserved during the fusion process, which both limits leakage of encapsulated materials into the surrounding medium and avoids dilution of the reagents by intake of fluid from the surrounding medium. Therefore the techinque may be suitable for quantitative studies of the mixing of chemicals and may prove to be a useful tool for combinatorial chemistry involving only femtoliters of reagents.

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